

**Genetically Biotinylated Recombinant Antibody in Immunofiltration Assay
by Light Addressable Potentiometric Sensor for Identification
of Venezuelan Equine Encephalitis Virus**

5 This Application claims the benefit of U.S. Provisional Application No. 60/456,939, filed on March 25, 2003, the entire content of which is incorporated by reference in this application.

FIELD OF THE INVENTION

10 This invention relates to the construction of a genetically biotinylated single chain fragment variable (scFv) antibody against Venezuelan equine encephalitis virus (VEE) being applied in a system consisting of an immunofiltration-enzyme assay (IFA) with a light addressable potentiometric sensor (LAPS) for the rapid identification of VEE. The IFA/LAPS assay averts the conventional method of employing antibodies which were
15 biotinylated chemically.

BACKGROUND OF THE INVENTION

List of Prior Art Literatures

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Venezuelan equine encephalitis virus (VEE), belonging to alphavirus genus of the family *Togaviridae*, is associated with encephalitis (Johnson et al., 1968; Franck et al., 1970). Although natural infection with this virus results from mosquito bites, the virus is also highly infectious by aerosol (Johnston et al., 1996). Thus, VEE is a potential
10 biological warfare / bioterrorist (BW/BT) agent of concern. The use of biological agents as BW/BT weapons is not a new concept, and alleged uses of biological agents as military or terrorist weapons can be found throughout history (Christopher et al., 1997). The most recent case of use of the biological agent, anthrax, as a bioterrorist agent occurred in USA in 2001 (Blendon et al., 2002). Identification of biological agents
15 involves either finding the agent in the environment or medical diagnosis of the agent and/or its effect(s) on human or animal victims. Early and rapid detection and identification of a biological agent is crucial for immediate and specific treatment of affected individuals and in order to limit the epidemic spread of associated disease.

Antibodies are critical reagents used in several biodetection platforms for the
20 identification of biological agents (Libby et al., 1989; Lee et al., 1993; Gehring et al., 1998; Lee et al., 2000; Carlson et al., 2000; Emanuel et al., 2000). Monoclonal antibody (mAb) 1A4A1 is specific for an envelope glycoprotein of VEE. This MAb has been shown to be highly reactive to VEE (Roehrig et al., 1985). Single chain variable fragment (scFv) mAbs are comprised of variable regions of heavy and light chains of
25 immunoglobulin, covalently connected by a peptide linker (Huston et al., 1988). These

small proteins generally retain the specificity and affinity for antigen similar to their parental mAbs and possibly bind to poorly accessible epitopes more efficiently due to their small size (Marin et al., 1995; Bruyns et al., 1996). The attractiveness of scFv antibodies is that they can be produced economically and stably in bacteria and can be manipulated via genetic engineering for different purposes, such as biotinylation (Luo et al., 1998). Recently, an anti-VEE mAb116 scFv Ab was cloned from 1A4A1 MAb (as taught in US Patent Application No. 10/096,246, herein incorporated by reference). This scFv showed an affinity to VEE comparable to that of the parental 1A4A1 MAb and therefore is a good candidate as immunodiagnostic reagent.

Previously, a flow-through immunofiltration-enzyme assay (IFA) in conjunction with a light addressable potentiometric sensor (LAPS) has been used for identification of microbial agents (Libby et al., 1989; Lee et al., 1993; Gehring et al., 1998; Lee et al., 2000). However, the IFA/LAPS system described to date utilized biotin-streptavidin-mediated capture filtration of immunocomplexes in which one of the analyte-specific antibodies required chemical biotinylation. The process of chemical biotinylation is commonly associated with impairment of the antigen-binding site on the antibody. Other disadvantages of chemically biotinylating mAbs as immunodiagnostic reagents include the cost and time required for growth and maintenance of hybridoma cell lines and production and purification of mAbs, and the potential for the occurrence of genetic mutation during repeated cycles of cell growth, rendering routine production of mAbs from hybridoma cell lines difficult, expensive, and time-consuming. Chemical biotinylation of antibodies is also time-consuming. Most biotins bind to amino groups on proteins and the degree of labeling tends to differ from batch to batch. The possibility

also exists that the biological activity of the antibody may be negatively affected by the labeling procedure (Miralles et al., 1991).

Accordingly, it is desirable to eliminate the need for chemical biotinylation. In US Provisional Application No. 60/448,902 submitted earlier (which is herein
5 incorporated by reference), the present inventors genetically fused a gene encoding a streptavidin-binding peptide to an anti-VEE scFv antibody gene. The resulting recombinant fusion antibody not only retained VEE antigen-binding specificity similar to that of the parental mAb, but also possessed streptavidin-binding activity. It would be advantageous to employ the genetically fused biotinylated scFv antibody in the
10 IFA/LAPS assay in order to obviate the disadvantages of using antibodies prepared by chemical biotinylation.

SUMMARY OF INVENTION

The present invention teaches the sensitivity of the IFA/LAPS assay for
15 identification of VEE, when genetically biotinylated scFv (B-scFv) antibody is used. It also compares such sensitivity between the chemically biotinylated parental mAb and those which were genetically biotinylated.

It is an object of the present invention to investigate the precision, accuracy, and specificity of the IFA/LAPS assay incorporating genetically B-scFv antibody for the
20 identification of VEE and to demonstrate that such assay is highly sensitive, specific, quantitative and reproducible.

It is another object of the present invention to show the potential for batch-to-batch variability resulting from inequality in the number of biotin molecules labeled per antibody molecule, when biotinylated chemically.

According to the invention, it provides a method for detecting VEE using a
5 genetically B-scFv antibody, comprising: (a) reacting the genetically biotinylated scFv Ab with a sample containing VEE for observing antigen-binding activity; and (b) analyzing the reactant by a system consisting of an immunofiltration-enzyme assay with a light addressable potentiometric sensor.

10 **BRIEF DESCRIPTION OF THE DRAWINGS**

Figure 1 is a schematic representation of the IFA/LAPS system.

Figure 2 shows standard curves for IFA/LAPS assay incorporating with biotinylated scFv (B-scFv) and fluoresceinated polyclonal Ab (F-pAb) in the detection of VEE.

Figure 3 shows quantitation of VEE by IFA/LAPS assay incorporating B-scFv. Each
15 data point represents the mean (n=6) of an independent determination of VEE concentration.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

Materials and Methods

20 Reagents and solutions

Sodium dihydrogen phosphate ($\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$) and Triton X-100 were obtained from BDH Chemicals (Toronto, ON). Bovine serum albumin (BSA), sodium chloride (NaCl), sodium hydroxide (NaOH), Tween-20 and urea were obtained from Sigma-

Aldrich Canada Ltd (Oakville, ON). Streptavidin was obtained from Scripps Laboratories (San Diego, CA). Biotinylated BSA-block nitrocellulose membrane sticks and antibody labeling reagents (*N*-hydroxysuccinimide esters of carboxyfluorescein and dinitrophenyl biotin) were purchased from Molecular Devices Corp (Menlo Park, CA). Anhydrous dimethylformamide (DMF) was purchased from Biolynx Inc (Brockville, ON). Sephadex G-25 columns were obtained from Amersham Pharmacia (Baie d'Urfé, QC). Washing buffer consisted of 10 mM NaH₂PO₄ (pH 6.5), 150 mM NaCl, and 0.05% Tween-20. Assay buffer consisted of 10 mM NaH₂PO₄ (pH 7.0), 150 mM NaCl, 0.025% Triton X-100, and 0.1% BSA. The substrate solution was 100 mM urea in the wash buffer (pH 6.5).

Viruses

VEE strain TC-83 and Western equine encephalitis virus (WEE) strain B11 were prepared as follows. Viruses were cultured in Vero monkey kidney cells or in baby hamster kidney cells (America Type Culture Collection, Rockville, MD). Cells were maintained in complete Dulbecco Minimum Essential Medium (DMEM) (Invitrogen Canada Inc., Burlington, ON) containing 5% fetal calf serum. When cells were confluent, the medium was decanted and replaced by a volume of virus inoculum, in DMEM, sufficient to just cover the monolayer. The virus inoculum was spread over the monolayer every 15 min and was allowed to adsorb to the cells for a total of 1 hr. Fresh complete medium was added and the flasks were incubated for several days to one week. When 90% cytopathic effect was reached, cells and medium were harvested and centrifuged at 10,000g for 30 min. To the pooled supernatants, polyethylene glycol (~6,000 MW) (7 % final concentration) and NaCl (2.3% final concentration) were added

and the mixtures were allowed to incubate with stirring for 15-20 hours at 4 °C. The mixtures were then centrifuged at 10,000g for 30 min and the resulting supernatants discarded. The virus pellet was resuspended in a small volume of phosphate buffered saline (PBS) and stored at -70 °C to await purification. The virus particles were purified
5 by sucrose density gradient centrifugation by standard techniques. Samples were centrifuged at 100,000g at 4 °C for 3.5 hr. Fractions containing virus were pooled and the pooled virus was dialyzed against PBS. Protein concentration of the purified virus was determined by protein assay and virus purity was assessed by polyacrylamide gel electrophoresis.

10 Antibodies

Genetically biotinylated scFv antibody to VEE was constructed from mAb 1A4A1 to VEE, expressed in bacteria and purified in accordance with the method disclosed in US Provisional Application No. 60/448,902. The hybridoma cell line producing 1A4A1 mAb was provided by Dr. J.T. Roehrig (Division of Vector-borne
15 Infectious Diseases, Centers for Disease Control and Prevention, Fort Collins, CO). MAb 1A4A1 was purified from vectraCell™ bioreactor (BioVectra™ Diagnostic Chemicals Ltd., Charlottetown, Prince Edward Island) cultures using protein G affinity chromatography (Biolynx Inc.). Polyclonal antibody (pAb) to VEE was purified from rabbit serum using protein G affinity chromatography. Anti-fluorescein urease conjugate
20 was purchased from Molecular Devices Corp.

Instrumentation

The LAPS used in the present invention was the Threshold™ Unit marketed by Molecular Devices Corp. The instrument was controlled by a computer and Threshold™

version 2 software. Reactions took place on membrane sticks (Molecular Devices Corp.) and the ThresholdTM Unit could process a maximum of four membrane sticks simultaneously (eight reaction test spots per stick). Assay time was less than 2 hours.

Preparation of biotinylated mAb and fluoresceinated pAb

- 5 Biotinylated mAb (B-mAb) and fluoresceinated pAb (F-pAb) were prepared according to procedures outlined in the ThresholdTM manual. Briefly, antibodies were adjusted to 1 mg/ml in PBS. MAb 1A4A1 was biotinylated chemically via *N*-hydroxysuccinimide ester of dinitrophenyl biotin (reconstituted in DMF) for 2 hours at room temperature; the molar coupling ratio (MCR) of biotin hapten to antibody was 10:1.
- 10 The pAb was fluoresceinated via *N*-hydroxysuccinimide ester of carboxyfluorescein (reconstituted in DMF) for 2 hours at room temperature; the MCR of fluorescein hapten to antibody was 20:1. Unreacted haptens were removed using PD-10 columns (Amersham Pharmacia Biotech) equilibrated in PBS. The number of moles of hapten covalently bound per mole of antibody was determined for each conjugate using the
- 15 molar incorporation ratio (MIR) described in the ThresholdTM manual. The MIRs of B-mAb and F-pAb were 3.8 and 4.0, respectively. The labeled antibodies were stored at 4°C until used.

Assay procedure

The IFA/LAPS scheme for the ThresholdTM unit is depicted in Figure 1.

- 20 A volume of 100 µl of VEE (0-20 µg/ml) was incubated with 100 µl of genetically biotinylated scFv (B-scFv) or B-mAb (1-5 µg/ml) and 100 µl of F-pAb (1-5 µg/ml) at room temperature for 30 min. At the end of the incubation period, 100 µl (5 µg/ml in assay buffer) streptavidin was added to the reaction mixture and mixed

thoroughly. The resulting mixtures were filtered through the membrane sticks (mounted in filter bases) on the Threshold™ Unit under low vacuum (complete filtration in ~10 min). The sticks were washed with 2 ml wash buffer while filtering under high vacuum (complete filtration in ~8 min). One ml of anti-fluorescein urease conjugate was then
5 added to the membrane sticks and filtered under low vacuum, followed by the addition of 2 ml of wash buffer and filtration under high vacuum. The membrane sticks were removed from the filtration compartment, and inserted into the reader chamber containing the LAPS and the substrate solution, urea. The pH change with respect to time at the surface of the sensor was monitored as the rate of change of the surface potential with
10 respect to time in $\mu\text{V}/\text{sec}$. The rate of pH change depends on the number of urease-containing antibody-antigen sandwiches immobilized on the membrane stick during the filtration capture process.

Protein assay

Virus protein concentration was estimated spectrophotometrically with a micro
15 BCA protein assay kit purchased from Biolynx Inc.

Results

Optimal ratio between B-scFv or B-mAb and F-pAb for detection of VEE

A preliminary assay was first performed using a range of VEE concentrations (10
20 to 2,000 ng/spot) and antibody concentrations (ng/spot) in the following B-scFv/F-pAb ratios: 200:200, 200:100, 100:200, and 100:100. From the preliminary result, a VEE concentration of 500 ng/spot, providing a mid-range LAPS signal was chosen for further experiments. A series of IFA/LAPS experiments was performed to investigate the effect

of the ratios of B-scFv and F-pAb on the output response of the assay. The aspect of the response of interest was the signal to noise ratio (S/N). S represented the output of LAPS from VEE. N was the background of the assay, that is, the output of the LAPS for the reagents without VEE. The S/Ns obtained using varying B-scFv/F-pAb concentrations (ng/spot) with a constant concentration of VEE antigen (500 ng/spot) were determined (Table 1). The B-scFv/F-pAb ratio giving the highest S/N was found to be 250:500 (ng/spot). Similar experiments were performed to determine the optimal concentration ratios of B-mAb and F-pAb. The B-mAb to F-pAb concentration ratio giving the highest S/N with fixed concentration of VEE was, similarly, found to be 250:500 (ng/spot) (Table 1).

Standard curve and sensitivity

To prepare IFA/LAPS standard antigen concentration curves, two-fold serial dilutions of VEE ranging from 125 to 2,000 ng/spot (1.25 to 20 µg/ml) in assay buffer were titrated using optimal ratios of B-scFv or B-mAb and F-pAb (250:500 ng/spot) (Figure 2).

Assay sensitivity, or limit of detection (LOD), was defined as the lowest concentration of VEE producing an output signal greater than the background plus three standard deviations of the background, i.e., the lowest concentration that could be measured accurately and precisely. The LOD of the IFA/LAPS incorporating B-scFv for detection of VEE was 0.29 µg/ml, while that incorporating B-mAb was 0.34 µg/ml.

Assay precision

Intra- and inter-assay variabilities were evaluated by testing three different VEE concentration samples (20, 5, and 1.25 µg/ml) in six assays performed together on the

same day and on 6 different days, respectively. Intra- and inter-assay coefficients of variation for the precision were determined by the ratios of standard deviations and means from six assays. These ranged from 15 to 19% and 18%, respectively (Tables 2 and 3).

5 Assay accuracy

The IFA/LAPS system incorporating B-scFv was assessed for accuracy in quantitation of VEE by comparing the protein concentrations measured by protein assay with the protein concentrations estimated by the IFA/LAPS standard curve. For five different test samples in which concentrations of VEE were estimated by protein assay to be in the range of 1.25-20 µg/ml, the mean difference between protein concentrations estimated by protein assay and by IFA/LAPS standard curve was about 10%. A linear regression analysis relating protein concentration estimated by protein assay with protein concentration measured by IFA/LAPS yielded a high correlation ($r^2=0.967$) (Figure 3).

Assay specificity

15 The specificity of the IFA/LAPS assay incorporating B-scFv was evaluated by challenging the assay with one other member of the alphavirus genus of the family Togaviridae, WEE. The results showed no cross-reaction in the assay between VEE and WEE (data not shown).

20 **Discussion**

The present invention applies genetically biotinylated scFv antibody against VEE in a commercially available assay system employing IFA/LAPS for rapid detection of VEE. The invention demonstrates the feasibility of replacing a murine mAb, biotinylated

chemically, with a scFv antibody, biotinylated genetically, as immunodiagnostic reagent in the IFA/LAPS system to detect VEE.

Before comparing the sensitivity of assays incorporating B-scFv and B-mAb in the IFA/LAPS system for detection of VEE, optimization of the concentration ratios of B-scFv or B-mAb and F-pAb was investigated and identified. These ratios are influenced by the type and affinity of the antibodies and are important aspects of the assay. Optimizations of assay conditions such as buffer formulation, temperature, incubation time, etc., were not undertaken as these had been previously determined and were available in the ThresholdTM manual. It was found that the optimal concentration ratios of both B-scFv and B-mAb and F-pAb were 250:500 (ng/spot). By using these concentration ratios, a standard curve relating IFA/LAPS signal and VEE concentration was constructed. Although signals in the IFA/LAPS assay incorporating B-mAb were two-fold higher than in the assay incorporating B-scFv, the background noise in the assay using B-mAb was twice as high as in the assay using B-scFv, resulting in similar LODs whether B-scFv or B-mAb were used. The LOD with B-scFv was 0.29 µg/ml, while that with B-mAb was 0.34 µg/ml. These results indicated that genetically biotinylated scFv could replace chemically biotinylated mAb in the IFA/LAPS system. As disclosed in US Provisional Application No. 60/448,902, it had been shown that the sensitivity of an enzyme-linked immunosorbent assay (ELISA) incorporating B-scFv and horseradish peroxidase (HRP)-conjugated streptavidin to detect VEE was about 0.2 µg/ml, a LOD value comparable to that obtained in the IFA/LAPS system incorporating B-scFv. The IFA/LAPS system however, has advantages over ELISA in that the IFA/LAPS assay system requires less time and effort to perform.

The present invention also investigated the precision, accuracy, and specificity of the assay in order to further evaluate the IFA/LAPS system incorporating B-scFv. A standard curve relating protein concentration to IFA/LAPS signal was used to accurately assess the concentration of VEE. Protein concentration, whether estimated by IFA/LAPS standard curve or by protein assay, differed by no more than 10%. Intra-assay and inter-assay precision varied by less than 20%. In addition, the specificity of the assay was confirmed by showing no cross-reactivity with WEE, a virus with a genetic background and a disease spectrum very similar to that of VEE.

In conclusion, the IFA/LAPS incorporating B-scFv was shown to be accurate, precise, reliable, reproducible, and sensitive for use in measuring VEE. More importantly, use of this assay obviates the need for chemical biotinylation of antibody, which often results in potential impairment of the antibody antigen-binding site and batch-to-batch differences in degree of labeling.

It is to be understood that the embodiments and variations shown and described herein are merely illustrative of the principles of this invention and that various modifications may be implemented by those skilled in the art without departing from the scope and spirit of the invention.

In addition, the List of Prior Art Literatures referred to in the Background of the Invention section is incorporated by reference herein.

Table 1.

Effects of concentration ratios of B-scFv or B-mAb and F-pAb on S/Ns of IFA/LAPS assays to detect VEE

B-scFv/F-pAb	S/N	B-mAb/F-pAb	S/N
(ng/spot)	(μ V/s)	(ng/spot)	(μ V/s)
125:500	5.63	125:500	6.37
250:500	8.78	250:500	7.60
500:500	4.80	500:500	3.18
125:250	5.82	125:250	6.11
250:250	6.50	250:250	5.22
500:250	4.51	500:250	4.86
125:125	3.43	125:125	3.89
250:125	5.14	250:125	5.02
500:125	4.70	500:125	3.38

Samples were tested in replicates of four.

Table 2.

Intra-assay precision of IFA/LAPS assay incorporating B-scFv to detect VEE

VEE	N	Mean	Standard deviation	Coefficient of Variation
($\mu\text{g/ml}$)		($\mu\text{V/sec}$)	($\mu\text{V/sec}$)	(%)
20	6	2156	309	15
5	6	844	160	19
1.25	6	243	38	16

The intra-assay precision was determined from the results of six replicates performed together on the same day for each VEE concentration.

Table 3.

Inter-assay precision of IFA/LAPS assay incorporating B-scFv to detect VEE

VEE	N	Mean	Standard deviation	Coefficient of Variation
($\mu\text{g/ml}$)		($\mu\text{V/sec}$)	($\mu\text{V/sec}$)	(%)
20	6	2076	370	18
5	6	918	175	18
1.25	6	240	43	18

The inter-assay precision was determined from the results of six assays performed on different days for each VEE concentration.